# Nucleic Acid Nanostructure Assisted Immune Modulation

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## Abstract

The rapid advances of nucleic acid nanotechnology have transformed our view of nucleic acids from key biological components to versatile building materials. By programming the specific molecular forces including Watson-Crick base pairing, hydrophobic interactions and protein binding, the physicochemical properties (e.g., size, shape, sequence, valency) of nucleic acid nanostructures can be engineered to control their interactions with specific biological components. Importantly, nucleic acids are intrinsic immune regulators that can initiate or suppress immune activation. In this brief review, we highlight recent advances in the design of nucleic acid-based nanostructures in modulating the immune system, focusing on the nanoparticle transport in the lymphatic system, interactions between signaling molecules and immune cells, and nanoparticle strategies to enhance, evade, deviate, or suppress the immune activation. Nucleic acid nanotechnology provides important avenues and opportunities for future immunotherapy.

Keywords: Immune Modulation, Nucleic Acid Nanotechnology, Adjuvant Formulation, Innate Immunity, Nucleic Acid Delivery.

## Introduction

The immune system plays important roles in both health and diseases. When functioning properly, a healthy immune system not only protects the host from pathogenic infections and the development of tumor, but also prevents the overreactive immune cells from attacking the body's own tissues, a process known to lead to the onset of autoimmune diseases.[1] The central theme of human immune system is to effectively discriminate self and non-self, that is, to recognize the molecular signals between malignant and normal cells and maintain the homeostasis of tissue or organism. Although the idea that approaches that fine tune the immune system can be harnessed for cancer or autoimmune diseases is not new,[2, 3] only recently has the immunotherapy become part of the standard of care in the treatment of certain types of cancers.[4, 5]

Nucleic acids are essential macromolecules encoding genetic information for all living organisms. Beyond their traditional roles as genetic polymers, nucleic acids are known for their versatile biological functions.[6, 7] Both natural and synthetic nucleic acids (e.g., plasmid DNA, siRNA, antisense DNA, DNAzymes) can regulate a broad range of biological functions through molecular binding, catalytic reaction, or gene interference, making them promising candidates for a broad range of biomedical applications.[7] To date there are at least nine nucleic acid-based medicines that have been approved by the U.S. Food and Drug Administration (FDA),[8-11] including aptamer,[8] antisense DNA,[10, 12-14] and siRNA.[14, 15] Nucleic acids are also important immune modulators which can stimulate or inhibit immune reactions.[16, 17] As one of the key elements in the detection of invading pathogens, nucleic acids can activate innate immunity and subsequently initiate adaptive immune responses, enabling efficient clearance of infections.[18] Consequently, immunostimulatory nucleic acids are often used as vaccine adjuvants (e.g., HepB-CpG vaccine[19]). On the other hand, immune inhibition or evasion are being actively pursued as inappropriate recognition of nucleic acids can trigger the detrimental pathology associated with autoimmune diseases,[20] which are characterized by abnormal attack of healthy tissues by immune cells. Therefore, the immune

responses accompanied by using functional nucleic acids must be strictly regulated to maximize the therapeutic efficacy while minimize the toxicity/side effects. Recent studies have found that engineering the sequences/structures, the quantity/valency, and the organ/cellular and subcellular locations of nucleic acids can specifically modulate the immune system with minimal side effects.[21]

Like many other therapeutic nucleic acids, immunomodulatory nucleic acids require delivery systems to protect them from enzymatic degradation, target immune organs/cells, and promote intracellular accumulation.[22] In fact, both immune stimulatory[23-26] and inhibitory nucleic acids[27, 28] have been demonstrated to be effective and safe when appropriate formulations/delivery systems were used to control their dose, kinetics, biodistribution, and cellular/subcellular entry. A substantial effort has been directed to the development of nanoparticles for the delivery of nucleic acids for immune modulation.[24-26] Nanoparticles protect the nucleic acids from enzymatic degradation, prolong their circulating half-life, and overcome multiple biological and molecular barriers. More importantly, synthetic nanoparticles are well suited to mimic the live pathogens in size, geometry and repetitive surface display, promoting specific interactions with the immune system when administered in vivo.[29]

Among the many different types of nanomaterials explored for delivering nucleic acids, the programmable nucleic acid-based nanostructures are emerging as unique and novel materials to modulate the immune reactions.[17, 30, 31] The unique self-assembling characteristics of nucleic acids are attractive in using them as building materials for higher ordered nanostructures.[32-34] In fact, nucleic acids, especially DNA are excellent building blocks in constructing three-dimensional nanostructures with unprecedent complexity and precision over size, shape and valency. Particularly, recent studies demonstrated that nucleic acid-based nanostructures can overcome multiple biological and molecular barriers associated with delivering the unformulated nucleic acids.[32, 35] Here we review the recent studies in the design of nucleic acids nanostructures for

immune system modulation. We discuss the critical roles of nucleic acids in innate immunity and factors that affect their efficacy in immune modulation. We also discuss the challenges and opportunities for nucleic acid nanostructures in both preclinical and clinical translational research.

**Nucleic acids in innate immunity**

In classical immune activation, the host uses two general mechanisms to recognize and eliminate foreign pathogens. The first mechanism is the innate immunity which triggers rapid, non-specific inflammatory responses when pathogen associated molecular patterns (PAMPs)[36-40] or damage-associated molecular patterns (DAMPs)[39, 41] are recognized. PAMPs are highly conservative and distinct microbial molecules that bind to pattern recognition receptors (PRRs)[39, 40] such as Toll-like receptors (TLRs) expressed by the innate immune cells. In contrast, DAMPs are endogenous danger molecules released from damaged or dying host cells. The innate immune responses are characterized by the induction of acute inflammation (e.g., secretion of proinflammatory cytokines, type I interferons and chemokines), initiation of phagocytosis, and maturation of antigen presenting cells (e.g., dendritic cells, B cells and macrophages). Upon activation, innate immune system initiates rapid (in hours), nonspecific defense responses via phagocytosis, intracellular killing, antigen presentation, and immune cell recruitment. The second mechanism acts through adaptive immunity, also known as acquired immunity or antigen-specific immunity, which triggers the production of antibodies and proliferation of effector lymphocytes, both of which are highly antigen specific. Adaptive immune responses require longer time to develop (in weeks), but they are believed to be more sophisticated and more efficient in clearing invading pathogens. Moreover, adaptive immune system can produce a long-lasting immunological memory which allows for a rapid and powerful response to recurrent infections. Innate immune signaling network often regulates the adaptive immune responses, as the magnitude, quality and duration of the antigen-specific immune responses strongly dependent on the immunological cues from innate immune system.[42]

Among many of the molecular entities that trigger the innate immunity, nucleic acids and their metabolites are one of the major PAMPs or DAMPs sensed and recognized by a number of transmembrane and cytosolic PRRs, including the endosomal Toll-like receptor family and cytosolic cyclic GMP-AMP synthase (cGAS) and RIG-I-like (retinoic acid-inducible gene-I-like) receptor family (**Figure 1**).[16, 18-20] Activation of these PRRs results in upregulating the expression of proinflammatory cytokines, chemokines, type I IFNs, co-stimulatory signaling molecules, and other uncharacterized antimicrobial proteins to initiate an effector immune response.

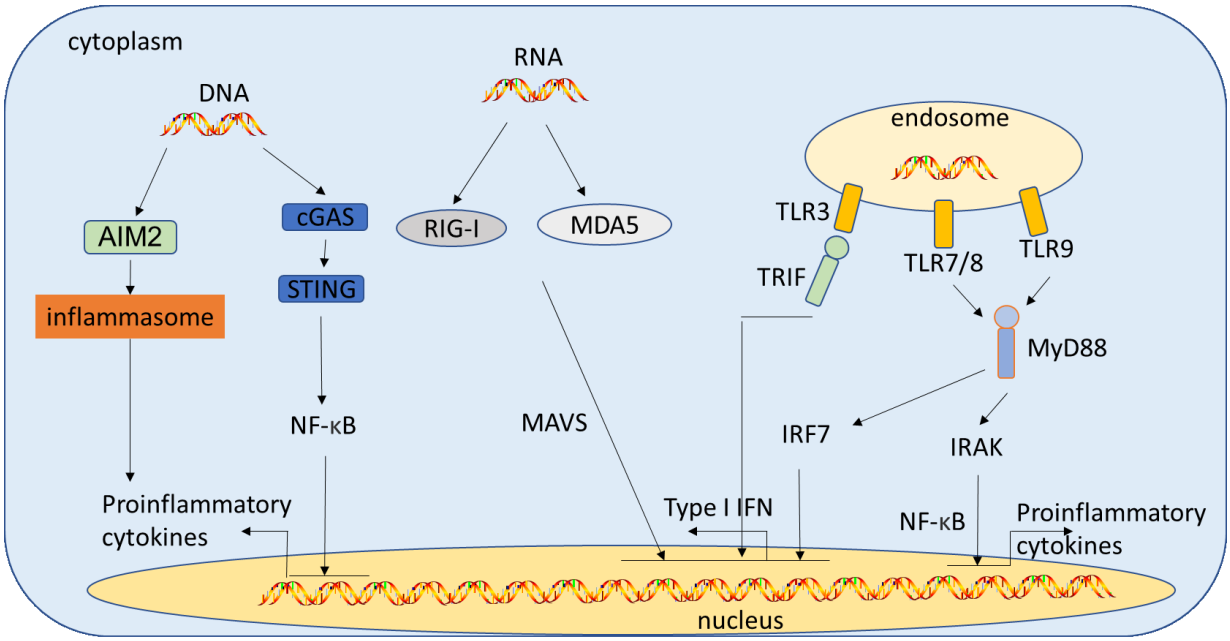


**Figure 1. Innate immune system activation by intracellular nucleic acids.** Nucleic acids are mainly detected by endosomal TLRs and other PRRs in the cytosol. TLR3, TLR7/8 and TLR9 are intracellular TLRs and recognize dsRNA, ssRNA and CpG DNA, respectively. Cytosol DNA sensing is primarily mediated by cGAS-STING and AIM2 inflammasome pathways. RIG-I and MDA5 are cytosolic RNA sensors which signal through MAVS and lead to the production of type I IFNs.

**Nucleic acid-sensing Toll like receptors.** TLRs are highly conservative transmembrane proteins expressed on the surface (extracellular) or intracellular compartments (intracellular) of

immune cells and play an important role in the innate immunity. TLRs recognize a wide range of molecular entities including nucleic acids derived from invading pathogens and endogenous tissues. Nucleic acid-sensing TLRs are predominantly confined in the intracellular vesicular compartments (e.g., endoplasmic reticulum, endosomes and lysosomes).[16, 18-20] However, activation only occurs within the endolysosomal compartments, as inhibition of endosomal acidification by known inhibitors prevents the TLR activation.[43] The restricted expression and action of these TLRs in endosomes is believed to minimize the risk factors for eliciting an unwanted immune activation by self-nucleic acids, as under normal circumstances host DNA is usually excluded from these locations.[16] The transmembrane Toll-like receptors TLR3, TLR7/8 and TLR9 recognize double stranded RNA, single stranded RNA (ssRNA) and single stranded DNA in the endosome, respectively. TLR3 recognizes double stranded RNA including viral RNA, synthetic RNA (poly I:C) and small interfering RNAs. TLR7/8 recognize guanosine- and uridine-rich single stranded oligoribonucleotides, certain small synthetic molecules such as imidazoquinolines and nucleosides/nucleosides analogs (**Figure 1**).[44, 45] According to its crystal structures, TLR7 possesses dual binding sites for ssRNA and small molecular ligands.[46] TLR8, however, has been found to recognize uridine and short oligonucleotides at distinct sites, suggesting that ssRNAs are not direct TLR8 ligands, instead, products derived from degradation of ssRNA bind and activate TLR8 synergistically.[47] Beyond direct stimulation on TLR7/8, certain sequences of oligonucleotides are able to differentially modulate the TLR7/8's immune stimulation by small molecular ligands.[48, 49] For example, thymidine-rich nucleic acids (e.g., thymidine homopolymer poly(dT)) were reported to inhibit the TLR7 and enhance TLR8 activation when immune cells were stimulated with imidazoquinolines, although poly(T) alone do not activate TLR7 or TLR8.[48, 49] TLR9 is the only DNA recognizing TLR in the endosomes. It recognizes unmethylated CpG (cytidine-phosphate-guanosine) motifs typically found in bacteria and viruses or synthetic DNA.[50] Small degradation products of CpG containing DNA, although incapable of

activating TLR9 alone, was recently found to augment the activation of TLR9 by CpG containing DNA.[51]

Depending on the adaptor proteins, TLRs act through two main pathways to initiate the downstream signal pathways, which ultimately lead to the production of NF-κB-dependent proinflammatory cytokines (e.g., TNF-α, IL-12) and type I interferons (**Figure 1**).[52] The activation of TLR7/8 and TLR9 recruit the adaptor protein MyD88, which in turn forms a complex with the members of IRAK kinase family, and releases NF-κB to induce proinflammatory gene expression.[52] In plasmacytoid dendritic cells (pDCs), the recruit of MyD88 also signals through IRF7,[53] leading to secretion of large quantities of type I IFNs in response to TLR7/8 or TLR9 activation (**Figure 1**).[53, 54] Structural differences in TLR ligands have been shown to induce differential cytokine responses through these two distinct signal pathways, favoring production of either proinflammatory cytokines or type I interferons.[55] It is also observed that the prolonged retention of the signaling complex in the early endosomes correlated with induction of IFNs.[56] The activation of TLR3 is MyD88 independent, instead, it relies on the association of adaptor protein called TRIF, which leads to production of type I interferons (**Figure 1**).[52, 57] TLR3 activation also leads to the production of proinflammatory cytokines via NF-κB.[52, 57]

**Cytosolic nucleic acid-sensing.** In addition to TLR-based nucleic acids detection in the endosomes, mammalian cells can detect nucleic acids in the cytosol through several receptors.[58, 59] Activation of these cytosolic receptors leads to the production of type I interferons and proinflammatory cytokines.

Cytosolic DNA sensing is primarily mediated by the cyclic GMP-AMP synthase-stimulator of interferon genes (cGAS-STING) pathway (**Figure 1**).[58, 60] cGAS is a DNA sensing nucleotidyl transferase enzyme which upon DNA binding, catalyzes the cyclization of ATP and GTP to form cyclic GMP-AMP (cGAMP). cGAMP is a secondary messenger which binds to STING and triggers the production of type-I interferons (IFNs) and proinflammatory cytokines and chemokines.[61, 62]

STING also can be directly activated by invading bacterial cyclic dinucleotides, such as cyclic diGMP, cyclic diAMP and bacterial cGAMP.[63, 64] Another pathway for cytosolic DNA sensing involves the absent in melanoma 2 (AIM2), which upon binding to DNA, forms a heterocomplex AIM2 inflammasome (**Figure 1**).[65-67] The formation of inflammasome leads to the production of proinflammatory cytokines such as IL-18 and IL-1β.[68] Interestingly, although both of the pathways react to intracellular DNA, activation of AIM2 inflammasome appeared to dampen the activation of STING, suggesting AIM2 pathway negatively regulates the STING activation.[69]

Cytosolic RNA sensing and immune activation in mammalian cells is dominated by RIG-I-like receptor (retinoic acid-inducible gene-I-like receptor, RLR) family, including RIG-I and melanoma differentiation associated gene 5 (MDA5).[58, 70] RIG-I binds short single- or double-stranded RNA ligands with a 5'-triphosphate end.[71, 72] In contrast, MDA5 binds longer genomic RNA.[73] Both receptors signal through mitochondrial antiviral signaling proteins (MAVS), leading to production of type I interferons.[58]

**Nucleic acid-based nanostructures**

Nucleic acids are ideal materials for building higher-order and complex nanostructures because of their highly predictable geometry and programmable interactions.[32, 35] For example, the diameter of DNA double helix is ~2 nm and a helical turn contains 10.5 base pairs (~3.4 nm) (**Figure 2a**).[74] Further, significantly complex nucleic acid-based nanostructures can be obtained by simply programming the Watson-Crick base pairing and/or hydrophobic interactions.[32, 35] These unique structural characteristics have made nucleic acids intriguing building blocks for creating a large structurally diverse architecture, including 1D wires, tubes, 2D patterns, crystals, and 3D DNA crystals/polyhedral (**Figure 2b**).[32, 35, 75, 76] These complex structures are typically assembled from rationally designed short nucleic acid segments (tiles). Another important development in the field is DNA origami, which involves the use of many short nucleic acid strands (staple) to fold a long nucleic acid into nanoscale two- and three-dimensional shapes and patterns

(**Figure 2c**).[76] The detailed design and biological applications of these self-assembled nucleic acid nanostructures have been extensively reviewed recently, and the interested reader is referred to relevant references.[32-35, 77] In addition to the above nanostructures with well-defined size and shape, various nucleic acid nanostructures have been assembled from the rolling circle amplification (RCA), which usually produces nanostructures with undefined size.[78, 79]

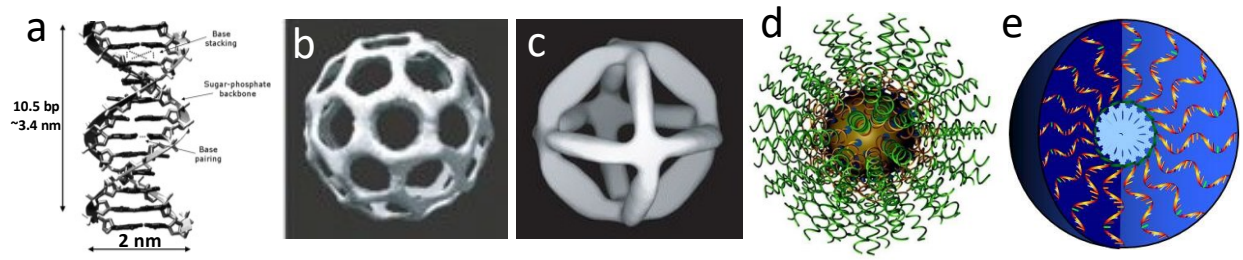


**Figure 2. Nucleic acids and their representative 3D self-assemblies.** (a), Double stranded nucleic acids. Reprinted with permission from ref 74. Copyright © 2011, Springer-Verlag Berlin Heidelberg. (b), DNA buckyball (~80 nm diameters) self-assembled from rationally designed DNA motifs. Reprinted with permission from ref 75. Copyright © 2008, Copyright © 2008, Springer Nature. (c), DNA octahedron (~22 nm diameters) folded from a 1.7 kb ssDNA. Reprinted with permission from ref 76. Copyright © 2004, Springer Nature. (d), Spherical nucleic acids formed by immobilizing oligonucleotides on gold surface. Reprinted with permission from ref 80. Copyright © 2012, American Chemical Society. (e), Nucleic acid micelles self-assembled with lipid-conjugated oligonucleotides. Reprinted with permission from ref 83. Copyright © 2010 WILEY‐VCH Verlag GmbH & Co.

Another important category of nucleic acid-based nanostructure is the spherical nucleic acid (SNA) nanoparticles, defined as highly oriented, well organized three-dimensional (3D) spherical arrangement of short nucleic acids (**Figure 2d**).[80] The 3D configuration of SNAs dramatically changes the physical and chemical properties of nucleic acids which enhance stability against nucleases, promote their cellular entry without the need for transfection agents, and improve

biocompatibility in animal models.[80, 81] SNAs have become versatile tools for the targeted delivery of functional nucleic acids. A similar 3D spherical architecture is the oligonucleotide micelle assembled from lipid-oligonucleotide conjugates (**Figure 2e**). However, because oligonucleotide micelles are self-assembled by the non-covalent hydrophobic interactions, the structure integrity of these surpramolecules relies on a combination of factors, including molecular weights of both ODN corolla and lipid core, the presence of cations which alleviates electrostatic repulsion from oligonucleotides, and the presence of biological components such as cells and serum proteins.[24, 82, 83] In a complex biological environment where cells and serum proteins are present, there exists a delicate three-way equilibrium between the intact micelles, the albumin-binding state, and the membrane-inserted state.[24] Notably, the stability, and subsequent interactions with the biological surroundings of these ODN micelles can be fine-tuned by simply controlling the lipid-ODN's molecular structures.[24] For example, in the presence of serum proteins, when a long diacyl lipid (≥ 16 carbons) was used for conjugation, the micelles dissemble themselves and bind tightly to serum albumin which serves as an excellent endogenous carrier for targeted nucleic acids delivery.[24] A portion of the lipid-ODN also spontaneously insert into membrane bilayers in the presence of cells.[24, 82, 83]

## Nucleic acid nanotechnology in immune modulation

**Nucleic acid-based nanostructures for lymph nodes targeting.** Lymph nodes are secondary lymphatic organs where the immune cells, such as macrophages, dendritic cells, B cells and T cells interact to initiate adaptive immune responses. Immunostimulatory signals must reach antigen presenting cells in the lymph nodes, activate both the innate and adaptive immune system, and persist for a sufficient time to prime B and T cells. Recent studies have shown that targeting the immune cells in the lymph nodes is an effective strategy for immune modulation.[84-87] Several design parameters affect the fate of peripherally injected materials, including size, surface charge, hydrophilicity and interactions with endogenous components present in the connecting tissues.

[84-87] The walls of lymphatic capillaries are composed of partially overlapped endothelial cells, which are permeable to particles less than 100 nm.[87, 88] In contrast, tight junctions of blood capillaries form a tight seal between neighboring endothelial cells which restricts the permeation of molecules.[88] Although lymphatic capillaries are more permeable than blood capillaries, small particles (< 5 nm) are preferentially absorbed into the blood as there are about 10 times more blood capillaries than lymphatic capillaries in the interstitial area under skin dermis.[87, 88] The unique structure of lymphatic capillaries allows the permeation of large size materials such as proteins, cells and other cell debris to the lymphatic system. After lymphatic entry, these particles are then filtered by the antigen presenting cells in the lymph nodes. In addition to size, particle charge also plays important role in the lymph node drainage and retention. While positive charge of the particles facilitates immune cell uptake and LN retention, it also hampers particle's ability to drain from the injection sites to draining LNs, as the interstitial matrix exhibits a net negative charge.[88, 89] Conversely, negatively charged particles showed improved lymphatic uptake compared with their positively charged or neutral counterparts. Finally, in situ protein binding and trafficking has been identified as an effective approach to target the draining lymph nodes after subcutaneous injection.[24]

Programming DNA nanostructures with controlled size, shape, charge, can facilitate the lymph node draining and subsequently elicit immune responses. Kim and coworkers constructed a DNA tetrahedron for sentinel lymph node imaging (**Figure 3a**).[90] The size of the self-assembled DNA tetrahedron (8.89 ± 0.22 nm diameters) was designed for optimal lymphatic drainage and lymph node retention. Compared with the linear DNA, DNA tetrahedron exhibited enhanced draining in the sentinel lymph nodes. Besides efficient lymph node draining, DNA tetrahedron showed enhanced cellular uptake, which resulted in prolonged retention in sentinel nodes. The enhanced lymph node accumulation might also explain the improved immunogenicity of protein-based vaccines delivered by DNA tetrahedron.[91]

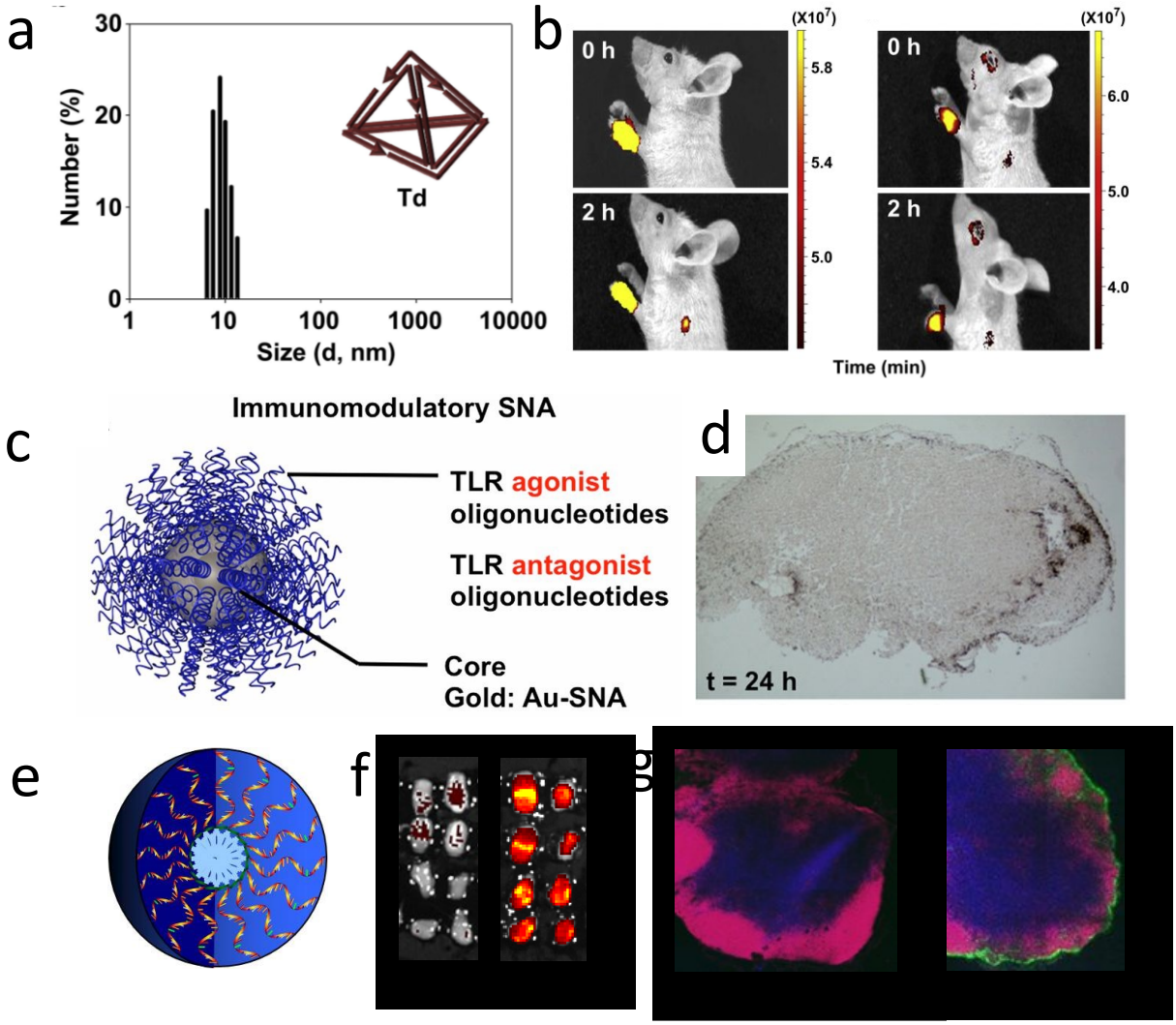


**Figure 3. Lymph node-targeting nucleic acid nanostructures.** (a and b), a DNA tetrahedron (~9 nm diameters) accumulated in the sentinel lymph node after subcutaneous injection. (a), Dynamic size scattering data of DNA tetrahedron. (b), IVIS fluorescent images of mouse after injection of tetrahedron (left) or linear DNA (right). Reprinted with permission from ref 90. Copyright © 2013 Elsevier Ltd. (c and d), Spherical nucleic acids with gold nanoparticle core (c) efficiently drained to lymph node (d). Reprinted with permission from ref 23. Copyright © 2015 National Academy of Sciences. (e-g), Oligonucleotide micelles accumulated in the draining lymph nodes after subcutaneous injection. (e) ODN micelles contain a hydrophilic DNA corona and a hydrophobic lipid core. IVIS fluorescence images (f, left: naked ODN; right: ODN micelles) and

Immunohistochemistry (g, left: naked ODN; right: ODN micelles) of draining lymph nodes 24 hours after injection. Reprinted with permission from ref 24. Copyright © 2014, Springer Nature.

Mirkin and coworkers showed that immunomodulatory nucleic acids can be targeted to draining lymph nodes in mice when they were organized into spherical nucleic acid form (**Figure 3b**).[23] The small molecular size (~20 nm) and negative charge of these SNAs facilitate the drainage and retention in the lymph nodes. Unlike the soluble nucleic acids, which have limited cell membrane permeability, SNAs are efficiently taken up by immune cells, leading to enhanced immunomodulatory activity in vitro and in vivo.[23]

Liu et.al. synthesized oligonucleotide-based micelles which upon subcutaneous injection, accumulate in the lymph node by binding to and trafficking with endogenous albumin protein (**Figure 3c**).[24] These oligonucleotide micelles are self-assembled from a diacyl lipid conjugated at the 5'-end of the oligonucleotide. In a complex biological fluid where cell and proteins are present, lipid-ODNs partition between self-assembled micellar state, membrane-anchoring state and albumin protein-binding state. Notably, lymph node accumulation was primarily caused by albumin-hitchhiking: amphiphiles bind and transported to the antigen-presenting cells in the lymph nodes.[24] Appropriate hydrophilic-lipophilic balance (HLB) appeared to be the key to shift the equilibrium toward albumin-binding state, which is critical in the lymph node draining and accumulation.[81]

**Nucleic acid nanostructures improve stability and cellular uptake.** Because nucleic acids are detected intracellularly, for an effective immune modulation, nucleic acids must be stable enough and must be able to enter the cells. While unmodified nucleic acids are susceptible to degradation in biological fluids, assembling nucleic acids into nanostructures protects them from rapid enzymatic degradation.[92, 93] Nucleic acid-based nanostructures have shown excellent nuclease stability and structural integrity in physiological settings by hiding the enzyme binding sites also by creating a 3D steric effect which restricts the enzyme access.[79, 94-96] In addition, unless a

transfection reagent is used, the high molecular weight and polyanionic nature have largely prevented the cellular entry of the vast majority of the unmodified nucleic acids. However, numerous nucleic acid-based nanostructures are efficiently taken up by a variety of cells without the aid of transfection agents.[35, 93, 97] For example, Turberfield's group proved that DNA tetrahedron (~7 nm) remain substantially intact within cells for at least 48 hours.[98] In this study, uptake of DNA tetrahedron was seen in cultured human embryonic kidney cells. Since DNA structures are highly negatively charged and lack the ability for membrane permeation, endocytosis was suggested to mediate the uptake in these cells.[98] Li et. al., demonstrated the enhanced stability of DNA tetrahedron in serum as well as in cells compared with DNA duplex.[31] Despite the high anionic charge of the DNA tetrahedron, efficient uptake was observed in macrophage-like RAW264.7 cells and Hela cells.[31] Using a series of inhibitors, the group found that the tetrahedron structures were rapidly internalized by the caveolin-dependent pathway.[99] Similar prolonged enzymatic stability and enhanced cellular uptake were observed with DNA origami nanostructures,[95, 100] rolling circle amplification-templated nanostructures,[79] and spherical nucleic acids.[80] Mirkin's group has systemically investigated the cellular entry mechanism of spherical nucleic acids.[101] They found the internalization was largely sequence independent, although SNAs with higher G content showed a higher degree of internalization.[102] Additionally, In C166 cells, SNAs appeared to bind strongly to class A scavenger receptors, which, in turn, promoted the internalization of SNAs via caveolae-mediated pathway.[101] Since the size of these structures ranges from a few nanometers (for DNA tetrahedron) to several hundred nanometers (for DNA origami), and the types of cells used in these studies differed dramatically, it is unlikely that the successful cellular entry can be attributed to a single, specific mechanism.

The stability and cellular entry can be further improved by adding additional modality to nucleic nanostructures. Coating the DNA nano-octahedron with PEGylated lipid-membranes further protected the DNA from nuclease digestion, prolonged the elimination half-life and bioavailability

in vivo.[103] Interestingly, shielding the nucleic acids also dramatically reduced the non-specific immune activation.[103] Hydrophobic modification is another way to enhance the cellular uptake for nucleic acids. Although the detailed mechanisms of action remain unclear, ODN micelles assembled from diacyl lipid-ODN conjugates were efficiently internalized in different types of cells in vitro and in vivo.[24, 82, 83, 104] Another strategy to enhance the cellular uptake in a target-specific manner is to conjugate nucleic acid-based nanostructures with small molecular ligands (e.g., folic acid),[105, 106] aptamer[107, 108], antibodies[109, 110] or peptides[111]. The presence of ligands enables the specific targeting certain cell populations (e.g., cancer cells or immune cells), while leaving the majority of other types of the cells intact.

Similar to other types of nanoparticles, the shape and arrangement of nucleic acid nanostructures affect their cellular uptake.[79, 112-115] DNA nanostructures with high aspect ratios appeared to favor the uptake by tumor cells but not phagocytic cells.[114] Ko and coworkers used DNA nanotubes as combinatorial vehicles for cellular delivery and observed significantly increased uptake.[116] Similar results were obtained when other DNA nanostructures were fabricated by rolling circle amplification.[79] Guo and coworkers studied the effects of size and shape and sequence of RNA nanostructures to the uptake and immunostimulation in macrophage like RAW 264.7 cells.[34, 112, 113] Their results clearly demonstrated that both the size and shape of RNA nanoparticles affect the immune activation, which correlated with the cellular uptake.

**Subcellular targeting by nucleic acid-nanostructures.** Pattern recognition receptors are expressed in different locations including plasma membrane surface and several subcellular compartments. Receptors that detect nucleic acids are primarily confined in the endolysosomal compartments and in the cytosol.[52] Targeting nucleic acid ligands to the locations of specific intracellular receptors can improve the therapeutic efficacy while minimizing toxicity associated with global immune activation. The diverse cellular entry pathways of DNA nanostructures have led to the different intracellular organelle accumulation after incubation.[93, 97] For example, DNA

tetrahedron was observed in the cytosol and lysosomes after internalization in HEK293 and HeLa cells, most likely due to the differences in their size and shape.[98] Similarly, the intracellular fates of origami structures varied according to their size, shape, and the cells used in the studies, with the majority of them traffic to cytosol and endolysosomal structures.[115] Both SNAs and lipid-based ODN micelles were found to traffic to and accumulate in the endosomes, with a small fraction escaped to the cytosol.[83, 104, 117] However, SNAs appeared to degrade primarily in the late endosomes without reaching lysosomes.[117] In contrast, lipid-ODN micelles were also observed in the lysosomes,[24, 83] suggesting the ODN structures and compositions play an important role in the intracellular trafficking.

**Nucleic acid-assisted receptor clustering**. Receptor clustering represents one of the important steps in the initial activation of a range of immune cells, including mast cells, basophilic granulocyte, B cells and T cells. Many pathogens exhibit highly ordered, repetitive antigens on their surfaces. This multivalent surface display is believed to effectively cluster antigen receptors and subsequently activate immune cells.[118, 119] Studies have demonstrated that spatial regulation of the antigen display affects the magnitude and quality of the immune activation.[118, 119] Nucleic acid technology can arrange ligands with sub-nanometer precision, thus offers unprecedent spacing and valency control in immune cells activation as well as mechanistic studies. Self-assembled DNA structures have been used to study the spatial requirement for clustering IgE receptors on mast cell surface.[120, 121] DNA are ideal for this purpose because of the highly predictable length and rigidity of DNA assemblies. Paar and coworkers characterized the separation distances of bivalent dinitrophenyl (DNP) in mast cell activation by using double stranded DNA oligomers.[121] It was found the activation signaling events were strongly dependent on the ligand spacing.[121] A subsequent trivalent DNA immobilized DNP with tunable spacing confirmed the kinetics and magnitudes of tyrosine phosphorylation and degranulation were ligand

spacing dependent: shorter length of ligand spacing (5 nm) was ~5-10-fold more potent than longer ligands (15 nm).[120]

Clustering cell surface transforming growth factor-β (TGF-β) by multivalent peptide ligands patterned on DNA nanostructures has been shown by LaBean and coworkers.[122] The use of DNA structures enhanced the sensitization of TGF-β signaling as compared to soluble ligands. In a recent study, Shaw et. al. precisely patterned antigens on DNA origami to study the effect of spatial distances on the antibody-antigen binding. They found that the binding affinities changed with spatial distances, which peaked at ~16 nm.[123]

The ability of nucleic acids in precisely controlling ligand valency and spacing can, in principle, be harnessed for B cell activation, since receptor clustering is an important mechanism triggering B cell activation.[124] Interestingly, to date no study aiming to use nucleic acid-based nanostructures for B cell activation has been published. In addition to multivalency and spacing, antigen mobility (flexibility) plays an important role in adapting appropriate ligand orientation for receptor binding and clustering.[125] Due to their relatively rigid nature, most static nucleic acid-based nanostructures lack the ligand flexibility and thus might not be the ideal biomaterials for this purpose.

Beyond direct immune cell activation, nucleic acids have been applied to investigate the kinetics and spatial reorganization of MHC-receptors in T cell signaling.[126] In this study, the extracellular domains of TCR and peptide-MHC were replaced with complementary strands of DNA. TCR-pMHC engaging and clustering were achieved and controlled by the mismatches on DNA hybridization. This elegant design revealed that a prolonged antigen presentation is required for T cell activation, suggesting TCR-pMHC stability is a significant factor governing the T cell signaling.[126]

**Nucleic acid-based nanostructures for immune activation or evasion.** One of the most studied immune stimulatory oligonucleotides is cytosine-phosphate-guanine (CpG)

oligonucleotide, a single-stranded synthetic ODN with CpG motifs that stimulate the TLR-9.[52, 127] CpG motifs can be readily integrated into nucleic acid-based nanostructures. Unlike the most of other nucleic acids, naked CpG ODN can be internalized by certain immune cells, although the degree of cellular uptake was limited.[128] This is because several cell surface receptors were found to bind and transport CpG into cells. Lahoud et. al. discovered that DEC-205, a lectin receptor expressed in a variety type of cells including dendritic cells, was a cell surface receptor for CpG oligonucleotides.[129] Moseman and coworkers revealed mannose receptor 1 was involved in CpG ODN uptake and trafficking.[128] Tanegashima and coworkers found that DCs uptake of CpG was dramatically improved in the presence of CXCL14.[130] Although the exact surface receptors for CXCL14 remain elusive, they concluded that CDCL14 formed complex with CpG and promote the uptake and endosome/lysosome transport.[130] In an attempt to augment the immune stimulatory effect of CpG ODN, Takakura's group in 2008 demonstrated that integrating CpG ODN into double stranded, Y-shaped structure significantly increased the uptake and immune activation in murine macrophage-like TLR9-positive RAW264.7 cells.[131] Further study revealed that higher ordered assemblies such as dendrimer-like DNA induced greater cytokine productions in macrophage-like cells, suggesting the immune activation is at least in part, determined by the DNA structure complexity, which is observed to correlated with more efficient internalization.[132] Based on these observations, the same group also constructed a series polypod-like structures and found that the increasing the pod number increased the immunostimulatory activity (**Figure 4a**).[133] Fan's group constructed DNA tetrahedron incorporating multiple CpG motifs and demonstrated the greatly enhanced immunostimulatory effect compared with unmodified CpG (**Figure 4b**).[31]

Guo and coworkers systemically studied the immunostimulatory activities of CpG delivered by RNA nanostructures. In their 2014 publication they compared the in vitro and in vivo (mouse) immune stimulation of CpG incorporated in RNA triangles (~10 nm), squares (~12 nm) and

pentagons (~15 nm) and found the degree of immune stimulation critically depended on the size, shape and CpG valency of RNA structures (**Figure 4c**).[112] The number of CpG (valency) and the size of planer polygons were found to correlate with immunositmulation in mouse macrophage-like RAW 263.7 cells.[112] Subsequent studies by the same group revealed that the immunostimulations were sequence dependent.[113] Additionally, 3D RNA structures showed stronger immunostimulation than planer structures, demonstrating the highly tunable ability of RNA nanostructures for immune modulation.[34, 113]

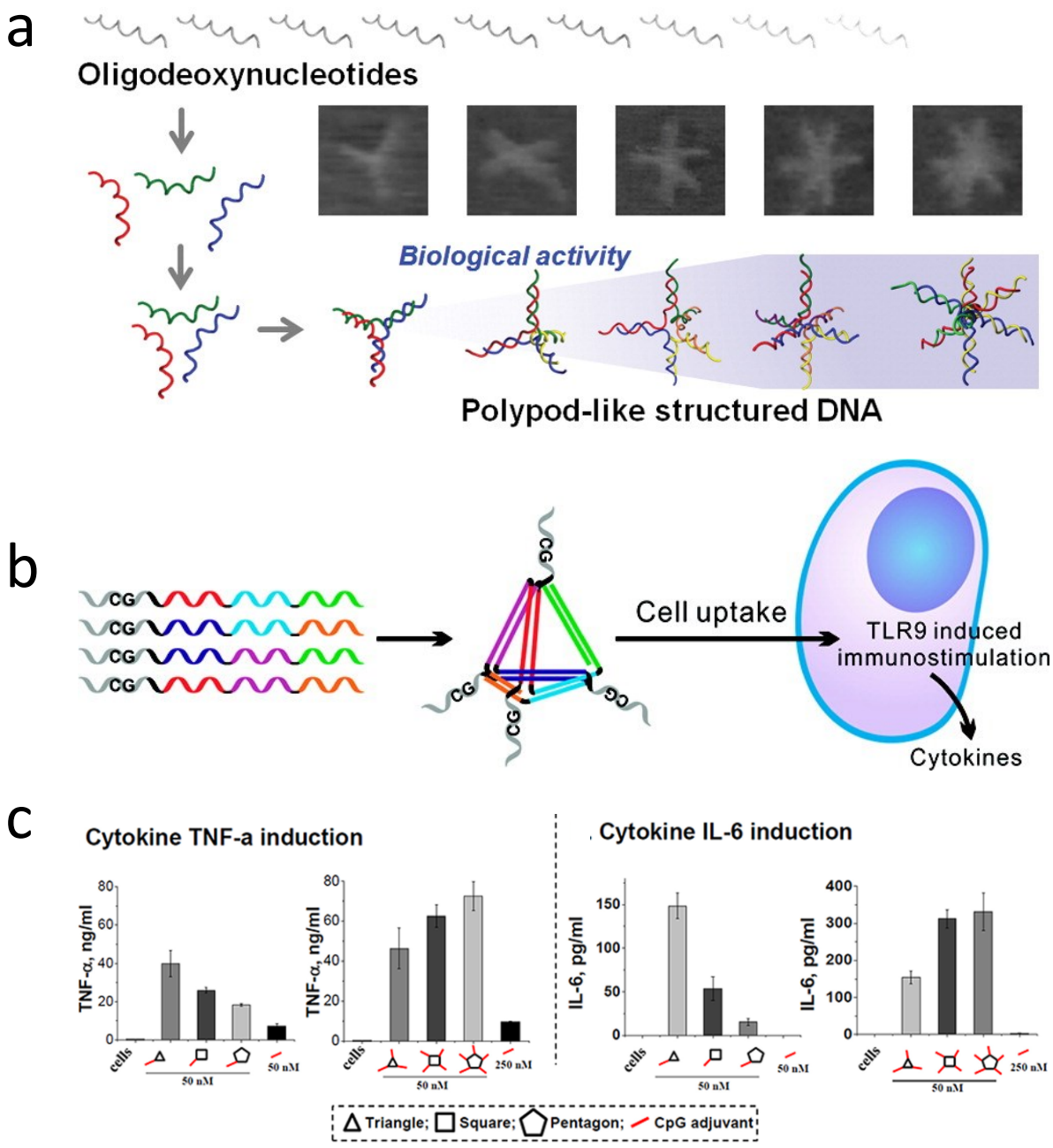


**Figure 4. Delivery of immunostimulatory CpG by different nucleic acid nanostructures.** (a), polypod-like DNA nanostructures mediated CpG delivery and immune activation. Reprinted with permission from ref 133. Copyright © 2012, American Chemical Society. (b), Multiple CpG ODN-bearing tetrahedron for immunostimulatory ODN delivery. Reprinted with permission from ref 31. Copyright © 2011, American Chemical Society. (c), Effect of RNA-CpG polygons on cytokine

induction in RAW 264.7 cells. Reprinted with permission from ref 112. Copyright © 2014, Oxford University Press.

Recently Afonin's group systemically studied the immunological recognition of 25 representative RNA and DNA nanostructures in peripheral blood mononuclear cells (PBMCs).[134] Their results showed that nanostructures based on nucleic acids were intrinsically immunogenic. However, the uptake as well as the immunostimulatory activities (monitored by measuring type I and III interferons) were only observed in the presence of lipofectamine 2000, a transfection agent which facilitates the uptake and perhaps the subcellular organelle targeting.[134] It was not immediately clear why in this study all the nucleic acids nanostructures varying with size, shape and compositions did not internalize in human PBMCs in the absence of a delivery carrier. However, it is known that the uptake and cellular distribution of nanoparticles depend on cell type (primary vs. immortalized, cancer vs. healthy cells). Nevertheless, the overall immunostimulation was correlated with nanoparticles' shape and composition.[134] Additionally, plasmacytoid dendritic cells were identified as the major producers for interferon production. Further mechanistic study revealed that endosomal TLR especially TLR7 signaling are essential for the recognition of these immunostimulatory nucleic acid nanoparticles.[135] The short nucleic acids degraded from the nanostructures could, at least in principle, directly activate or boost the activation of TLRs, or stimulate cytosolic receptors such as cGAS-STING.[21] Further research is needed as there is currently no study address these possibilities.

In contrast to immune activation, immune evasion is needed for many therapeutic nucleic acids where immune activation is considered as an unwanted side effect. It was suggested that the host immune response to nucleic acid-based nanostructures can be controlled by tuning their in vivo stability,[136] which can be leveraged by enzymatic ligation, and chemical modification (2'-OMe base modification[137]). It is also important to point out that not all nucleic acid-based nanostructures are equally immunogenic. Thus, one could speculate that the immunogenicity of nucleic acid

nanostructures might be mitigated by engineering the nucleic acid sequences, chemical modification, size, shape, and stability.[105, 136]

These elegant studies demonstrated that by tailoring the physicochemical properties such as size, shape, and valency of the nucleic acid nanostructures the immune responses can be fine-tuned to respond to a variety of different situations. Both immune activation and evasion are possible, providing a programmable approach for immune modulation for functional delivery of nucleic acid-based therapeutics.

**Nucleic acid-based nanostructures for Immune deviation and suppression.** Beyond immune activation or evasion, immune deviation or suppression is also a key requirement in treating allergy/autoimmune diseases or organ transplantation. Immune deviation refers to the phenomenon that the induction of humoral immunity prevents the subsequent induction of cellular immunity or vice versa, while the immune suppression refers to approaches to dampen the overreactive responses against self-tissues (autoimmune diseases) or organ transplants. Some allergic disorders, such as asthma and rhinitis are triggered by T-helper 2 (Th2) cell-mediated immune responses following exposure to allergens or environmental antigens.[138] The TLR-9 ligand CpG ODN adjuvants are known to skew the immune responses from T-helper 2 toward Th1 polarization.[139] Vaccination with allergens combined with CpG ODN was shown to suppress Th2-mediated cytokines accompanied with a Th1-biased immune deviation.[139] Immunomodulation with CpG ODN without allergens has also been shown to be effective in protecting experimental asthma upon allergen challenge in sensitized mice.[140]

Immune suppression has long been sought to dampen autoimmunity or to reduce the rejection of transplanted tissues. Immune inhibitors are routinely used to reduce inflammation in diseases. Because inappropriate activation of TLRs can lead to the initiation and/or perpetuation of inflammation and autoimmunity, TLRs and their signaling pathways have emerged as potential therapeutic targets.[141] For example, TLR activation by endogenous or exogeneous ligands

triggers the rapid production of inflammatory cytokines and has been associated with a variety of autoimmune diseases such as systemic lupus erythematosus and psoriasis.[142] Antagonistic nucleic acids have been thus developed as TLR inhibitors to prevent or treat autoimmune diseases.[143] Inhibitory oligonucleotides with different sequences have been identified and their suppressive activities have been described in vitro and in vivo.[143, 144] Although the detail mechanisms of their activities are not fully understood, it is believed that most of these inhibitory ODNs block TLR7/8 and/or TLR9 activation, which subsequently dampen the inflammatory reactions.[143] In addition, TLR independent mechanisms have also been described. For example, oligonucleotides with telomeric repeats (e.g., A151) are potent immune suppressors signaling through signal transducer and activator of transcription (STAT) 1, 3, and 4,[145] or through mammalian target of rapamycin (mTOR) pathways.[146]

Similar to the immunostimulatory nucleic acids discussed above, delivery of suppressive nucleic acids benefited from nucleic acid-based nano carriers. The spherical nucleic acids developed by Mirkin and coworkers have been applied for the delivery of immunoregulatory ODNs.[23] A TLR9 antagonist oligonucleotide, 4084F[147] was incorporated in SNAs. Thanks to its 3D structure, immunoregulatory 4084F SNA showed approximate 8-fold increase in potency in TLR9 inhibition when compared with soluble 4084F in RAW-Blue macrophages. Administration of 4084F in SNA form, but not in soluble form, inhibited the production of NF-κB and TNF-α. More importantly, SNA delivery of 4084F demonstrated enhanced antifibrotic activity in a mouse model of nonalcoholic steatohepatitis.[23]

Yu and coworkers tested the potential treatment of autoimmune diseases or chronic inflammation of a lymph nodes-targeting suppressive ODN.[28] Synthetic suppressive A151 containing repetitive TTAGGG motif was engineered with an albumin-binding diacyl lipid at the 5'-terminal. Subcutaneous injected amphiphilic A151 accumulated in the draining lymph nodes and exhibited potent inhibition of TLR9-elicited CD8 T cell and B cell responses in vivo.[28]

In addition to TLR suppression, nucleic acid nanostructures carrying decoy ODN has been tested. Afonin and coworkers designed smart responsive nucleic acid nanofibers and polygons that contain DNA duplexes encoding NF-kB decoys.[148] These NF-kB ODNs are embedded in separate nanostructures and upon conditional activation, reform the functional NF-kB decoy which binds to and inhibits the expression of NF-kB function in living cells.[148]

**Current issues in the field**

**Structure-based rational design.** Although the self-assembled nanostructures of nucleic acids can be precisely programmed, rational design of these nanoparticles with desired physicochemical properties to overcome the multiple biological barriers for immune modulation remains an unmet challenge.[33, 35] Given the diverse structural features of nucleic acid-based nanostructures, it is unknown whether there is a uniform and optimal formulation in order to maximize the immune modulatory efficacy. It is unlikely that a comprehensive screening and assessment can fulfil all the requirements for delivery of immune signals in vivo for different diseases. Consequently, it remains to be determined whether a structure with specific composition, shape, or size would simultaneously possess sufficient stability, immune system targeting, cellular and intracellular permeation, and retention. At this stage, structure-based rational design remains a significant challenge in the field.

**Limited loading ability of the nucleic acid-nanostructures.** Unless a hybrid system[103, 149] is used, the therapeutic agents that can be loaded with nanostructures constructed by pure nucleic acids are currently limited to nucleic acids or their intercalators (e.g., doxorubicin). Therefore, a large number of the immune modulators (e.g., TLR agonists and antagonists) do not have a loading mechanism to associated with nucleic acids-based nanostructures. In contrast, the encapsulation techniques enable the loading of hydrophilic, hydrophobic, and amphiphilic drugs in traditional polymeric nanoparticles.

In addition to drug loading ability, a comprehensive evaluation of the quantity, and quality of the cellular uptake, trafficking is needed. Many studies have indicated that nucleic acid-based nanostructures can enter cells without the need for transfection agents. However, many of these studies used fluorescence signals to measure and track the intracellular fates of nucleic acids structures. Recent studies suggested that better guidelines are needed as intracellular fluorescence cannot be correlated with the cellular uptake and the integrity of the nucleic acid structures.[150] In another study, efficient cellular internalization and immune stimulation of a variety of nucleic acid nanostructures were observed only in the presence of appropriate transfection agents.[134] These studies suggest cautionary guidelines must be implemented in the field.

**Limited number of clinical studies.** Nucleic acid-based nanostructures have emerged as a research tool in biomedical applications including bioimaging, biosensing, immune modulation, and drug delivery. However, the clinical translation rate is low. Although therapeutics based on nucleic acids are generally considered to be safe, their potencies in the innate immune activation have raised concerns of non-specific inflammation which limits their clinical applications. It is known that repeated CpG stimulation leads to syndromes related to cytokine storm in mice,[151] and that formation of anti-CpG antibodies have been detected in clinical studies.[152] In general, comprehensive engineering approaches that control the dose, timing and localization are needed to minimize these side effects.[84, 153] Further, although cellular and animal models have greatly advanced our understanding of immune functions in health and disease, the direct translation from the results obtained in cellular or animal studies to humans remain difficult because there are fundamental differences between humans and the animals in the immune system. For example, most of the above studies use CpG ODNs to demonstrate the principle of immune signal delivery by nucleic acid-based nanostructures. Although CpG ODNs are potent adjuvant in preclinical studies, the usefulness and data interpretation of using CpG DNA as vaccine adjuvant in animal models and are caveats because 1), the TLR-9 protein expressed by humans and mice

differs by 24% at the amino acid level;[154] 2), the cells that express TLR-9 vary between these species[155] and 3), the CpG ODN sequences that optimally stimulate immune cells differ between mice and humans. To date, spherical nucleic acids are the only nucleic acid-based nanostructures to reach clinical translation.[156] A phase Ib/II clinical trial (NCT03684785) is currently conducted by Exicure Inc. to evaluate the safety, tolerability, pharmacokinetics, pharmacodynamics and preliminary efficacy of intratumoral AST-008 (an SNA formulation of TLR9 agonist) in advanced solid tumors.[156]

**Conclusion**

The past decade has witnessed tremendous progress in the construction and biological applications of nucleic acid-based nanostructures. The ability to precisely control the size, shape, geometry and valency of nucleic acid-based nanostructures has attracted tremendous research interests for their biological applications. The unique physicochemical features of the programmable nucleic acid nanostructures have enabled them to resist to enzymatic degradation, prolong the circulating time, accumulate in the target tissue, increase their membrane permeation, and traffic to specific subcellular compartments. Consequently, incorporation of drug molecules, particularly immune modulators into nucleic acid nanostructures represents a new and novel approach for targeted drug delivery. The recent success of several immunotherapies against cancer is revolutionizing cancer treatment. As we gain more insights into the fundamental aspects of immunology in diseases, new and powerful nucleic acid-based structures and novel design principles will emerge. In the future, these immune signals might be combined with other treatment modalities, producing an optimal outcome for patients.

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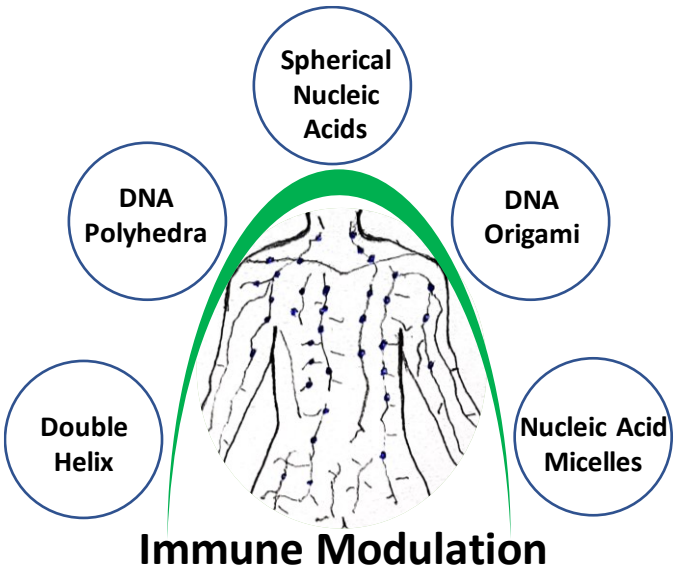
TOC graphic


Spherical Nucleic Acids
DNA Polyhedra
DNA Origami
Double Helix
Nucleic Acid Micelles
Immune Modulation